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A method for preparing PCR products for restriction-enzyme-directed cloning without enzymic digestion

Roy P. Dunford and Tristan A. Dyer

John Innes Centre, Norwich, Norfolk, UK NR4 7UH

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▼Annealing and ligation of complementary cohesive ends is one of the most efficient methods for joining DNA fragments. Various methods involving the joining of cohesive ends have been devised for cloning PCR products. TA cloning involves the annealing of an overhanging 3′ A residue on the PCR product with a 3′ T residue on specially prepared vector (Ref. 1). However, this method relies on the ability of certain thermostable DNA polymerases to add a 3′ A to PCR products. Some DNA polymerases used for PCR [e.g. Pfu polymerase (Stratagene)], do not have this feature and cannot be used for TA cloning. Other methods, such as ligation-independent cloning (LIC; Ref. 2, 3) and UDG cloning (Ref. 4), require the synthesis of extended oligonucleotide primers and the enzymic treatment of vector DNA to create compatible ends, both processes incurring added expense. Ligation of PCR products to cohesive ends generated by restriction enzymes may be desirable for some applications. Existing methods to achieve this rely on the use of extended PCR primers containing the appropriate restriction site followed by post-PCR digestion of the products. However, this approach is limited by the inefficient performance of restriction enzymes close to the end of DNA molecules (Ref. 5) and the possible presence of recognition sites within the PCR product.

Here we describe an improved method for preparing PCR products for ligation with vector DNA digested with restriction enzymes that generate cohesive termini. It is based on the recently described 'hetero-stagger' cloning strategy (Ref. 6). Briefly, the original method involved the generation of DNA fragments with 3′ CCC overhangs by the use of extended primers during PCR. These molecules are then annealed and ligated to a vector cloning site with 3′ GGG overhangs. The modification described here results in the creation of cohesive ends compatible with any restriction enzyme. An illustration of the procedure resulting in the generation of cohesive ends with 5′ overhangs is shown (Fig. 1s). Two PCR amplifications are carried out with different primer pairs. The first comprises a forward primer specific for the target sequence with a 5′ extension corresponding to the desired cohesive end and a reverse primer specific to the target sequence alone. The second pair contains forward and reverse primers with the same target-specific sequences as the first pair but with the reverse primer having a 5′ extension specifying a cohesive end. Amplification products are mixed in equimolar amounts, heat denatured and re-annealed at room temperature for 1 h. After re-annealing, four products are expected (see Fig. 1). Only duplexes formed by the association of single strands with the appropriate 5′ extensions (25% of the annealed products) can be ligated with vector digested with the corresponding restriction enzyme.

We tested the practical application of this method by subcloning the 5′ portion of a cDNA encoding the Calvin cycle enzyme sedoheptulose-1,7-bisphosphatase. Primer pairs were designed to amplify the appropriate region and generate 5′ and 3′ cohesive ends corresponding to those created by *Nde*I and *Esp*I, respectively. The primer extensions were also designed to recreate the appropriate restriction site on ligation, although this may not be required in all cases and can be avoided if necessary. PCR was carried out using the high-fidelity thermostable DNA polymerase Pfu (Stratagene) (see Ref. 7). Re-annealed amplification products were

Corresponding author: roy.dunford@bbsrc.ac.uk

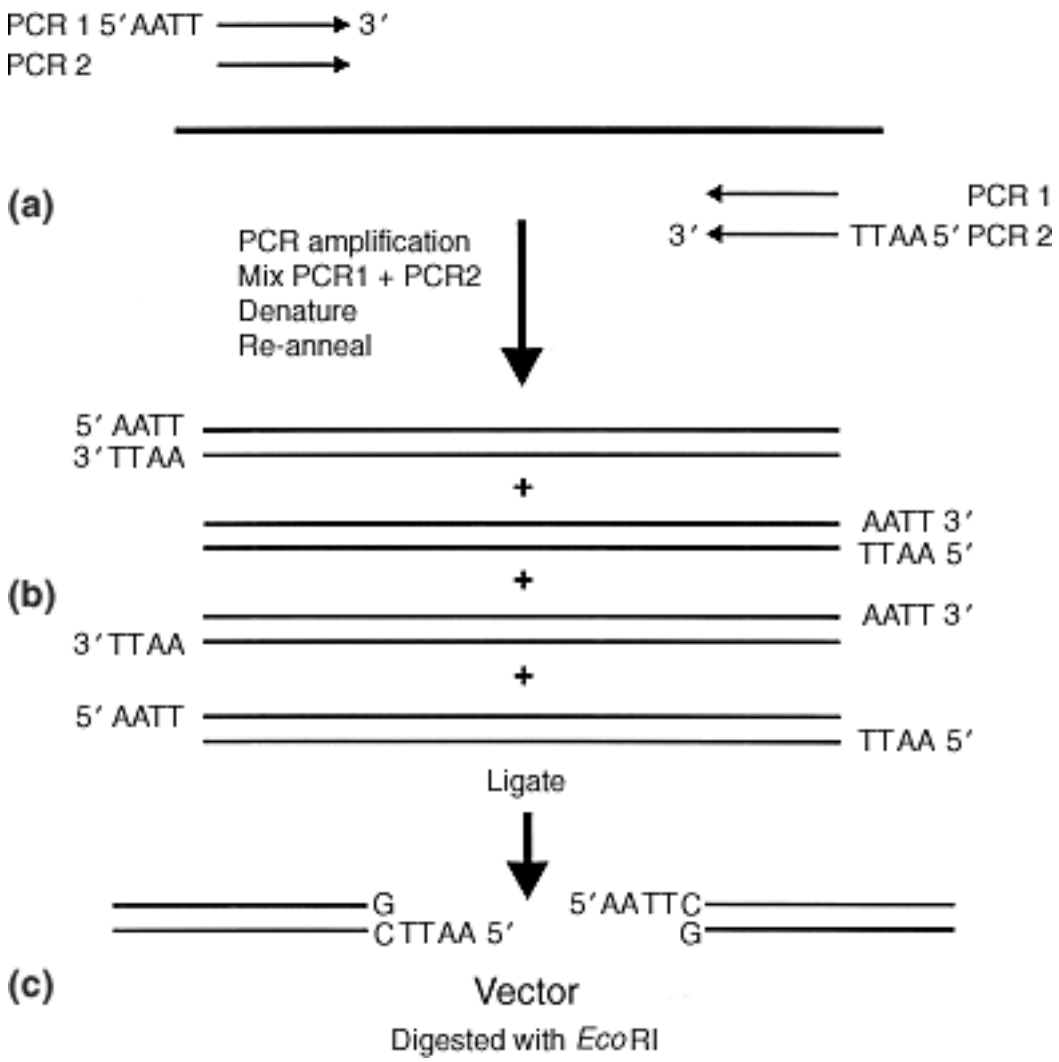

FIGURE 1. Outline of method for the generation of PCR products with cohesive ends compatible with those produced by restriction enzymes. (a) Primer pairs (short arrows) used to amplify a specific region from the target sequence (solid bar). Primers used in the two separate amplifications are labelled PCR 1 and PCR 2, respectively. The extension sequence TTAA-5′ specifies formation of a cohesive end compatible with that generated with the restriction enzyme *Eco*RI. (b) Duplexes formed after mixing, denaturing and reannealing the products of PCR 1 and PCR 2. Note that the amplified target sequence is identical in each case. (c) Only the last product has the correct 5′ overhangs and can be ligated to vector DNA digested with *Eco*RI.

ligated to the expression vector pET19b (Novagen) digested with *Nde*I and *Esp*I and subsequently transformed into *Escherichia coli* strain DH5*α* by electroporation. Plasmid DNA was recovered from the transformants using standard procedures and the presence of an insert of correct size with the expected ends was confirmed by restriction digestion.

In principle, this method should be successful with any restriction enzyme generating a defined 5′ or 3′ overhang. The use of high-fidelity thermostable DNA polymerases which do not add additional nucleotides to amplification products should ensure the creation of the correct cohesive ends for ligation (Ref. 7).

The advantages of this method include the ability to define the ends of cloned fragments by careful primer design, resulting in the retention or ablation of restriction sites. This may be desirable for maintenance of reading frame and specific codons for correct protein expression or for subsequent cloning steps. Furthermore, a relatively simple treatment, i.e. restriction digestion, is used to prepare vector DNA with cohesive ends. Finally, this method is not affected by the presence within PCR products of restriction sites identical to those used for cloning.

References

1 Holton, T.A. and Graham, M.W. (1990) *Nucleic Acids Res.* 19, 1156.
2 Aslanidis, C. and Jong, P.J. (1990) *Nucleic Acids Res.* 18, 6069–6074.
3 Haun, R.S., Serventi, I.M. and Moss, J. (1992) *BioTechniques* 13, 515–518.
4 Nisson, P.E., Rashtchian, A. and Watkins, P.C. (1991) *PCR Methods Appl.* 1, 120–123.
5 Kaufman, D.L. and Evans, G.A. (1990) *BioTechniques* 9, 304–306.
6 Liu, Z. (1996) *Nucleic Acids Res.* 24, 2458–2459.
7 Cline, J., Braman, J.C. and Hogrefe, H.H. (1996) *Nucleic Acids Res.* 24, 3546–3551.

Products Used

Pfu DNA polymerase: Pfu DNA polymerase from Stratagene
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expression vector pET19b: expression vector pET19b from Novagen